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Inhibitory effect of 1α,25-dihydroxyvitamin D_3 on the growth of the renal carcinoma cell line

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Inhibitory effect of 1α,25-dihydroxyvitamin D_3 on the growth of the renal carcinoma cell line. We studied the effect of vitamin D compounds on the growth of the human renal carcinoma cell line (KU-2) and discovered a receptor protein specific for the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3. The KU-2 cell line was established from a pulmonary metastasis of renal cell carcinoma in a patient with hyperhemoglobinemia. The cells were tumorigenic in nude mice and clonogenic in a soft agar culture.

Vitamin D_3 derivatives suppressed proliferation of KU-2 cells in a monolayer culture and also clonogenicity in a soft agar culture dose–dependently. Of the vitamin D_3 derivatives tested, 1α,25-dihydroxyvitamin D_3 was the most potent in inhibiting cell growth, followed successively by 1α,24R,25-trihydroxyvitamin D_3, 25-hydroxyvitamin D_3, 1α-hydroxyvitamin D_3 and 24R,25-dihydroxyvitamin D_3 in that order. Analysis of the cell cycle phase of treated and non–treated KU-2 cells revealed that the action of 1α,25-dihydroxyvitamin D_3 was not phase–specific but simply extended the doubling time of the cells. Radioreceptor assay and sucrose density gradient analysis of the cytosol showed that KU-2 cells contained a 3.2S receptor protein to which 1α,25-dihydroxyvitamin D_3 was specifically bound (Kd = 20.8 ± 4.8 pM, Nmax = 87 ± 24 fmole/mg protein, 4000 molecules/cell). On the other hand, the equilibrium dissociation constant of internalization of 1α,25-dihydroxyvitamin D_3 (*Kint*) by intact KU-2 cells was 1.2 nM and the internalizing capacity was 33 fmole/8 × 10^6 cells (2500 molecules/cell) in the 10% serum medium, which was the same as that used in the growth study. This *Kint* value was very close to the half-maximal dose in growth inhibition. Also the affinity of various vitamin D_3 derivatives for binding to the cytosol receptor in the KU-2 cells was closely related to the ability to inhibit growth of the cells. These results indicate that the actions of vitamin D_3 derivatives in inhibiting proliferation and clonogenicity of KU-2 cells are affected by a receptor–mediated mechanism, and that the active form of vitamin D_3 may be one of the regulatory factors affecting the proliferation and other biological functions of renal carcinoma cells.

The importance of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the hormone of the parent vitamin D_3, in calcium metabolism, is well established. The major biological function of 1α,25$(OH)_2D_3$ is to enhance intestinal calcium transport and bone mineral mobilization by binding to a specific cytosol/nuclear receptor in those organs.

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Recently, much attention has been focused on the ubiquitous distribution of similar 1α,25$(OH)_2D_3$ receptors. The receptors have been found not only in such classical target organs of vitamin D as intestine and bone, but also in kidney, parathyroid, skin, pancreas, spleen, brain, ovary, and hematopoietic cells. It is also of great interest that the 1α,25$(OH)_2D_3$ receptors are found not only in normal cells, but also in several tumor cells. This suggests that the role of the hormone in biology extends far beyond its classical role in mineral metabolism.

In 1981, Abe et al [1] discovered that 1α,25$(OH)_2D_3$ inhibits proliferation and induces differentiation of mouse myeloid leukemia cells (M1) into monocyte–macrophages. Subsequently, Honma et al [2] reported that in vivo administration of 1α,25$(OH)_2D_3$ or 1α-hydroxyvitamin D_3 [1α$(OH)D_3$] considerably prolongs the survival time of syngeneic SL strain mice and athymic nude mice inoculated with M1 cells. The hormone also inhibits proliferation of the receptor containing human myeloid leukemia cells (HL-60) [3–5], human melanoma cells (Hs695T) [6], and rat osteosarcoma cells [7]. More recently, Dokoh et al [8] demonstrated that 1α,25$(OH)_2D_3$ inhibited the growth of receptor–containing rat osteosarcoma cells (ROS17/2.8) in a soft agar culture. Additionally, Wood et al [9] reported that the topical application of 1α,25$(OH)_2D_3$ suppresses papilloma formation induced by dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin. These results suggest the possibility that vitamin D compounds are useful as anticancer therapeutic agents.

In 1976, Katsuoka et al [10, 11] established a cultured cell line (KU-2) from a pulmonary metastasis of a renal cell carcinoma in a patient with hyperhemoglobinemia. Renal carcinoma tumors are known to be one of the least responsive tumors to anticancer chemotherapy. Recent therapeutic trials utilizing interferon or sex hormones have not yet achieved appreciable clinical efficacy. Therefore, more effective therapeutic modalities are needed to improve the prognosis of patients with renal carcinoma. All of these newly discovered principles regarding the relation of vitamin D_3 derivatives to cancer suppression and the presence of a receptor for 1α,25$(OH)_2D_3$ in the kidney [12, 13] led us to examine whether renal carcinoma cells possess similar 1α,25$(OH)_2D_3$ receptors and whether 1α,25$(OH)_2D_3$ inhibits the growth of such cells. The present study indicates that the renal carcinoma cell line KU-2 has a receptor protein

specific for $1\alpha,25(OH)_2D_3$ and that the hormone inhibits KU-2 cell proliferation by a receptor–mediated mechanism in vitro.

Methods

Cells and cell culture

The cultured cell line KU-2 was established by Katsuoka et al from a pulmonary metastasis of a renal cell carcinoma in a patient with hyperhemoglobinemia, after temporary heterotransplantation to a nude mouse in 1976 [10]. Electronmicroscopic features of the KU-2 cells are similar to renal tubular cells [14]. KU-2 cells are anchorage–independent in soft agar cultures and tumorigenic in nude mice [15]. The tumor from which KU-2 cells were derived has been maintained in nude mice so far and is confirmed to produce erythropoietin [16]. Cells were cultured in Eagle's minimum essential medium containing 1.8 mM Ca supplemented with 10% heat–inactivated fetal bovine serum (FBS, GIBCO, Grand Island, New York, USA), 0.292 μg/mliter of L-glutamine, 100 μg/mliter of streptomycin, and 100 U/mliter of penicillin G in a constant flow of 5% CO_2 and 95% air with humidity maintained at 37°C.

Effects of vitamin D_3 derivatives on cell growth

Effects of $1\alpha,25(OH)_2D_3$ and its derivatives on growth of KU-2 cells were studied in two different culture systems: a monolayer culture and a soft agarose culture. In the latter system [17], the feeder layer was made of 1 mliter of 0.5% agarose in culture medium in a 35 mm petri dish. The cells passaged two days before were detached from flasks by trypsinization and 1×10^4 separated single cells were inoculated in a culture layer made of 1 mliter of 0.33% agarose. Various concentrations of vitamin D_3 derivatives were added to both feeder and culture layers. The colony–forming rate was calculated from the number of colonies which consisted of 20 or more cells two weeks after inoculation.

The cell cycle of treated and non-treated cells was analyzed as follows: the cells passaged 48 hr before were cultured in the absence or presence of $1\alpha,25(OH)_2D_3$ for an additional 48 hr. Then the cells were harvested by trypsinization and dehydrated with graded concentrations of ethanol. The cells were stained with 0.5% propidium iodide and analyzed by flowcytometry. Cell populations in different cell cycle phases were determined using the method of Slater et al [18].

Preparation of $1\alpha,25(OH)_2D_3$ receptor from KU-2 cells

A cytosol fraction of KU-2 cells was prepared as described by Shiina et al [19]. Approximately 1×10^8 cells in the exponential phase of growth were detached from petri dishes with 0.1 M EDTA in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), washed with PBS and pelletted three times by centrifugation at 4°C. Then the cells were sonicated in a hypertonic buffer which consisted of 0.3 M KCl, 2 mM EDTA, 10 mM Tris/HCl, pH 7.4 and 0.5 mM dithiothreitol (KETD-0.3). The homogenate was centrifuged at 30,000 × g for 10 min at 4°C. The resulting supernatant was centrifuged again at 225,000 × g for 60 min at 4°C to yield a cytosol fraction. Protein concentration in extracted cytosols was determined by the method of Bradford [20].

For DNA-cellulose chromatography, a nuclear extract was prepared by the method of Chandler et al [21]. Approximately 3.6×10^7 cells were harvested by trypsinization and resuspended in 5 mliter of serum–free culture medium. Then the cells were incubated with 0.5 nM $1\alpha,25(OH)_2[^3H]D_3$ for one hr at 37°C in a shaking water bath, and tritium–labeled cells were rinsed three times in ice–cold PBS containing 1% FBS, followed by centrifugation at 500 × g for five min. The resulting pellet was resuspended in 5 mliter of a buffer containing 0.1 M KCl, 1 mM EDTA and 10 mM Tris/HCl (KET-0.1), and homogenized with Polytron (Kinematica, Switzerland). The homogenate was centrifuged again at 1,100 × g for 15 min, and the pellet was resuspended in 0.5 mliter of a hypertonic buffer containing 0.3 M KCl, 1 mM EDTA and 10 mM Tris/HCl (KET-0.3), and centrifuged again at 12,000 × g for five min. The supernatant was diluted with a buffer containing no KCl (KET-0) to 3 mliter in volume and applied to DNA-cellulose chromatography.

Studies on $1\alpha,25(OH)_2[^3H]D_3$ binding to the cytosol receptor

After the cytosol extract was diluted with KETD-0.3 buffer to achieve a 1 mg protein/mliter in concentration, 0.5 mliter of the cytosol solution (0.5 mg protein) was incubated with graded concentrations of $1\alpha,25(OH)_2[^3H]D_3$ (specific activity = 85 Ci/mmole, Amersham International plc., Bucks, United Kingdom) with or without 1,000-fold radioinert $1\alpha,25(OH)_2D_3$ for 60 min at 25°C. The bound $1\alpha,25(OH)_2[^3H]D_3$ was separated by the hydroxylapatite method described by Wecksler and Norman [22]. The incubation was terminated by immersing the incubation tubes in an ice bath and by adding immediately 0.5 mliter of 50% hydroxylapatite slurry suspended in 10 mM Tris/HCl-0.1 M KCl, pH 7.5. The pellets were washed three times with 10 mM Tris/HCl-0.5% Triton-X, pH 7.5. The final washed pellet was quantitatively extracted with 1 mliter of a mixed solvent of methanol:chloroform (2:1) two times, and the solvent was transferred to scintillation vials and evaporated to dryness. Six mliter of the scintillation cocktail (ACS II, Amersham, United Kingdom) was added to the vials and the radioactivity of the samples was counted for two min with an Aloka LC-900 liquid scintillation counter.

The affinity of various vitamin D_3 derivatives for the specific $1\alpha,25(OH)_2D_3$ receptor was determined by a competitive receptor assay. Aliquots of the cytosol (1 mg protein/mliter) were incubated with 0.25 nM $1\alpha,25(OH)_2[^3H]D_3$, which was a saturated concentration for the receptor in KU-2 cells, in the presence or absence of graded concentrations of various D_3 derivatives. The $1\alpha,25(OH)_2[^3H]D_3$ bound to the receptor protein was extracted and counted.

The internalization of $1\alpha,25(OH)_2[^3H]D_3$ was studied as described by Manolagas et al [23]. The cells in exponential phase of growth were harvested, washed and suspended in Eagle's MEM containing 10% FBS at 2×10^7 cells/mliter. Aliquots of 0.4 mliter (8×10^6 cells) cell suspension were incubated with graded concentrations of $1\alpha,25(OH)_2[^3H]D_3$ with or without a 100-fold excess of radioinert $1\alpha,25(OH)_2D_3$ for four hr at 37°C in a shaking water bath. The tubes were immersed in an ice bath and the cells were washed with an isotonic buffer. Then the cells were sonicated in KETD-0.3 buffer and ultracentrifuged as previously described. Finally, the radioactivity in the supernatant was counted.

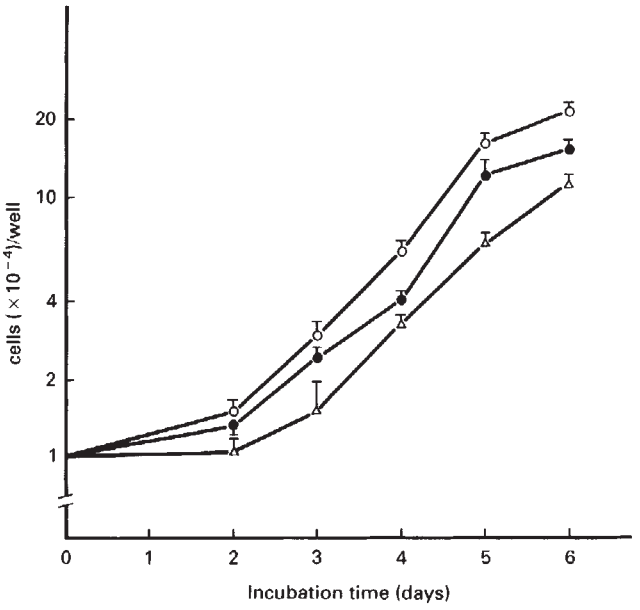

Fig. 1. *Growth curves of KU-2 cells in a monolayer culture treated with $1\alpha,25(OH)_2D_3$.* Symbols are: ○, control (0.1% ethanol); ●, 1.2 nM $1\alpha,25(OH)_2D_3$; △, 120 nM $1\alpha,25(OH)_2D_3$. Points and bars represent mean ± SD of three replicated determinations of triplicate experiments.

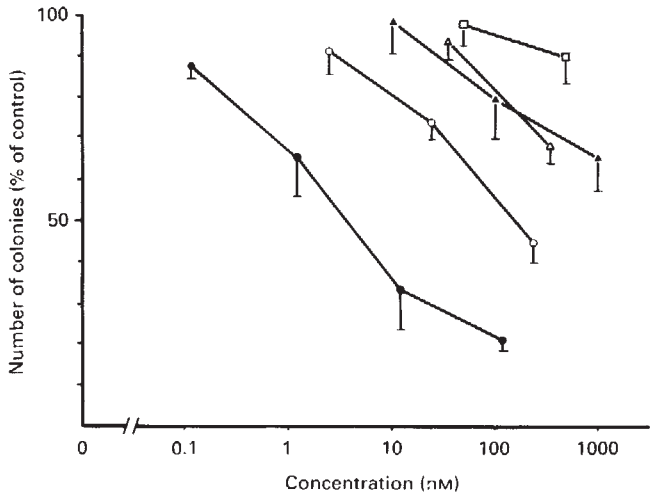

Fig. 2. *Dose response of suppression of the clonogenicity of KU-2 cells in a soft agar culture after incubation with graded concentrations of vitamin D_3 derivatives for 14 days.* Symbols are: ●, $1\alpha,25(OH)_2D_3$; ○, $1\alpha,24R,25(OH)_3D_3$; ▲, $25(OH)D_3$; △, $1\alpha(OH)D_3$; □, $24R,25(OH)_2D_3$. Points and bars represent mean ± SD of two replicated determinations of quadriplicate experiments.

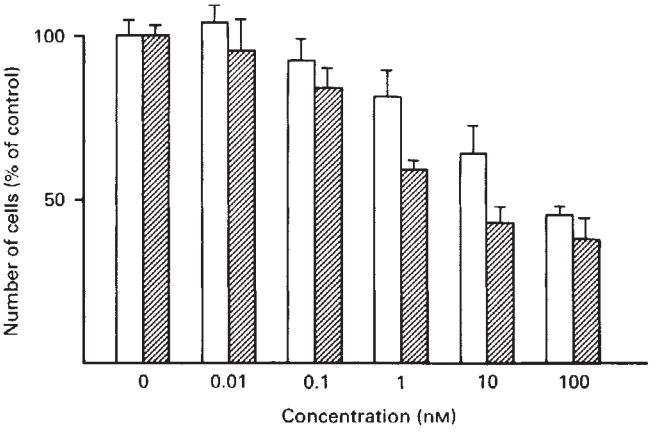

Fig. 3. *Effect of the FBS concentration added to the medium on the $1\alpha,25(OH)_2D_3$-induced suppression of KU-2 cell growth.* The cells were cultured in the 10% FBS medium (▭), or in the 2% FBS medium (▨) with graded concentrations of $1\alpha,25(OH)_2D_3$ for five days. Data are mean ± SD (represented by bars) of two replicated determinations of triplicate experiments.

Sucrose density gradient analysis

This analysis was performed as described elsewhere [24]. Aliquots (500 μliter) of the cytosol preparation of KU-2 cells were incubated with 1 mM $1\alpha,25(OH)_2{}^3HD_3$ in the presence or absence of a 200-fold excess of radioinert $1\alpha,25(OH)_2D_3$. After unbound radioligand was adsorbed by 50 μliter of dextran-coated charcoal, 200 μliter of the tritirum-labeled cytosol was layered onto a 4 to 20% discontinuous sucrose gradients prepared in KETD-0.3 buffer. After centrifugation at 208,000 × g (average force) for 18 hr at 4°C, 100 μliter fractions were collected from the top of the gradient tubes and radioassayed.

DNA-cellulose chromatography

DNA-cellulose chromatography was performed as described elsewhere [21]. The diluted nuclear extract (3 mliter) was applied to a 2.5 mliter DNA cellulose column equilibrated with KET-0.05. After washing with KET-0.05, the labeled receptor complex was eluted with a 20 mliter KET-0.05–KET-0.5 linear gradient. All operations were performed at 4°C. One mliter fractions were collected at a flow rate of 1 mliter/min, and the radioactivity of each fraction was counted. The salt concentration was determined with a conductivity meter.

Results

Effects on cell growth

$1\alpha,25(OH)_2D_3$ suppressed dose-dependent proliferation and clonogenicity of KU-2 cells in the presence of 10% serum. In the monolayer culture, the growth rate began to increase two days after inoculation, and the cells grew linearly up to day five or six. The average doubling time of the KU-2 cells was increased from 21.0 hr to 22.4 hr and 26.9 hr when the cells were exposed to 1.2 and 120 nM $1\alpha,25(OH)_2D_3$, respectively (Fig. 1). In the soft agar culture, $1\alpha,25(OH)_2D_3$ added to the semisolid medium also suppressed clonogenicity of KU-2 cells dose-dependently. The clonogenicity was reduced to 89.6 ± 2.8% of the control by adding 0.12 nM $1\alpha,25(OH)_2D_3$, to 65.0 ± 5.0% by 1.2 nM, to 30.0 ±8.0% by 12 nM, and to 20.7 ± 2.1% by 120 nM. $1\alpha,25(OH)_2D_3$ was the most potent in inhibiting clonogenicity, followed successively by $1\alpha,24R,25$-trihydroxyvitamin D_3 [$1\alpha,24R,25(OH)_3D_3$], 25-hydroxyvitamin D_3 [$25(OH)D_3$], $1\alpha(OH)D_3$ and 24R,25-dihydroxyvitamin D_3 [$24R,25(OH)_2D_3$] in that order (Fig. 2).

Suppression of KU-2 cell growth by $1\alpha,25(OH)_2D_3$ was enhanced by reducing the serum concentration in the culture medium to 2%. A half-maximal dose of $1\alpha,25(OH)_2D_3$ in inhibiting growth in a monolayer culture was approximately 0.2 to 0.3 nM in the 2% serum medium (Fig. 3), whereas a

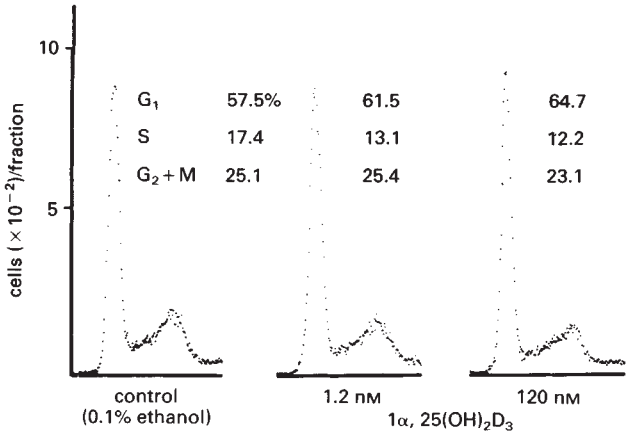

Fig. 4. *DNA histograms of KU-2 cells treated with 1α,25(OH)$_2$D$_3$.* KU-2 cells were incubated with 1α,25(OH)$_2$D$_3$ for 48 hr and analyzed by a flowcytometry to determine DNA contents. Cell populations in each cell cycle were counted from DNA histograms using the method described by Slater et al [17].

Table 1. Clonogenicity of KU-2 cells pretreated with 1α,25(OH)$_2$D$_3$

Concentration of 1α,25(OH)$_2$D$_3$	Plating efficiency, %
0 nM	45.7 ± 6.7
1.2	48.6 ± 6.1
12	50.4 ± 3.1
120	45.8 ± 5.5

KU-2 cells were treated with graded concentrations of 1α,25(OH)$_2$D$_3$ for 72 hr. Then the cells were dispersed, inoculated in a soft agar plate without 1α,25(OH)$_2$D$_3$ and incubated for 14 days. Data are mean ± SD of two replicated determinations of triplicate experiments.

half-maximal dose in the monolayer or the soft agar culture was approximately 2 to 3 nM in the 10% serum medium (Figs. 2 and 3).

The subpopulations of untreated KU-2 cells in each cell cycle phase were 57.5% in the G1 phase, 17.4% in the S phase and 25.1% in the G2 + M phase. DNA histograms of the KU-2 cells treated with 1α,25(OH)$_2$D$_3$ for 48 hr showed a decrease in the S and G2 + M peaks when each G1 peak was adjusted to the same peak height. The numbers of S phase cells decreased from 17.4% to 13.1 and 12.2% when 1.2 and 120 nM 1α,25(OH)$_2$D$_3$, respectively, were added. Consequently, the relative numbers of G1 and G2 + M phase cells to S phase cells increased. This indicates that 1α,25(OH)$_2$D$_3$ increases the G1 and G2 cells, or quiescent cells with the same DNA content, resulting in the extension of the doubling time (Fig. 4).

To determine the cytotoxicity of 1α,25(OH)$_2$D$_3$, KU-2 cells were pretreated with graded concentrations of the hormone for 72 hr. Then the cells were dispersed, inoculated in a soft agar plate without 1α,25(OH)$_2$D$_3$, and incubated for 14 days. The plating efficiency of the cells was not affected by the pretreatment with 1.2 to 120 nM gradations of 1α,25(OH)$_2$D$_3$ (Table 1).

Characterization of the receptor

Radioreceptor assay for the cytosol of KU-2 cells showed the presence of a receptor protein to which 1α,25(OH)$_2$D$_3$ was bound specifically. Scatchard plot analysis of the specific 1α,25(OH)$_2$D$_3$ binding showed a low equilibrium dissociation constant. Three replicates of the receptor assay and its Scatchard plot analysis showed that the dissociation constant (*Kd*) was 20.8 ± 4.8 pM and the binding capacity (*Nmax*) was 87 ± 24 fmole/mg protein (mean ± SE), which means 4000 binding sites/cell (Fig. 5).

To examine the role of the receptor in the mechanism of action of 1α,25(OH)$_2$D$_3$-induced growth suppression, we measured the specific uptake of 1α,25(OH)$_2$[^{3}H]D$_3$ by intake KU-2 cells under the same condition as used in the growth study. Cells were monitored for the specific internalization of various concentrations of 1α,25(OH)$_2$[^{3}H]D$_3$ in the presence of 10% serum in the culture medium. The results indicated that internalization by intact KU-2 cells was a specific and saturable process. Scatchard plot analysis of the specific internalization showed that the equilibrium dissociation constant for 1α,25(OH)$_2$D$_3$ internalization (*Kint*) was 1.2 nM and the internalizing capacity was 33 fmole/8 × 10^6 cells (2500 molecules/cell) (Fig. 6).

Sucrose density gradient analysis showed a sharp single peak of a macromolecular radioligand sedimented at 3.2S which was eliminated by adding 200-fold radioinert 1α,25(OH)$_2$D$_3$ (data not shown).

Analysis for the receptor in nuclear extracts using DNA-cellulose chromatography revealed the presence of a 1α,25(OH)$_2$D$_3$-binding component which was adsorbed to DNA at low ionic strength and was eluted at a concentration of 0.22 M KCl. This elution pattern is characteristic of the 1α,25(OH)$_2$D$_3$ receptor previously reported [5, 13, 21] (Fig. 7).

Relationship between binding affinity and growth inhibition

A study of competition of various vitamin D$_3$ derivatives for the specific 1α,25(OH)$_2$D$_3$-binding sites in the KU-2 cytosol showed that the concentrations of 1α,24R,25(OH)$_3$D$_3$, 25(OH)D$_3$, 1α(OH)D$_3$ and 24R,25(OH)$_2$D$_3$ required to displace 50% of the bound 1α,25(OH)$_2$[^{3}H]D$_3$ were about 6-,375-, 700- and 1,550-fold higher, respectively, than that of 1α,25(OH)$_2$D$_3$. The ability of the vitamin D$_3$ derivatives to inhibit proliferation of KU-2 cells was closely related to their affinities for binding to the 1α,25(OH)$_2$D$_3$ receptor in KU-2 cells (Fig. 8).

Discussion

The present study indicates that 1α,25(OH)$_2$D$_3$, even in physiological concentrations, inhibits proliferation and clonogenicity of KU-2 cells in vitro in a dose-dependent manner. As stated in the introduction, recent reports have demonstrated that 1α,25(OH)$_2$D$_3$ inhibits proliferation of several tumor cell lines. The present report adds a renal carcinoma cell line (KU-2) to the list of cells whose growth is suppressed by 1α,25(OH)$_2$D$_3$. The suppressing effect of 1α,25(OH)$_2$D$_3$ on KU-2 cells was confirmed in two different in vitro systems: a monolayer culture and a soft agar culture. The rate of growth suppression by 1α,25(OH)$_2$D$_3$ was higher in the latter than in the former. Though this may be related in part to the time of exposure to the vitamin, it may result alternatively from suppression of a clonogenic factor or a decrease in the malignancy of the cells.

The effect of 1α,25(OH)$_2$D$_3$ in inhibiting growth of KU-2 cells appears to be a function of a receptor-mediated mechanism. KU-2 cells were found to possess a 3.2S cytosol protein to

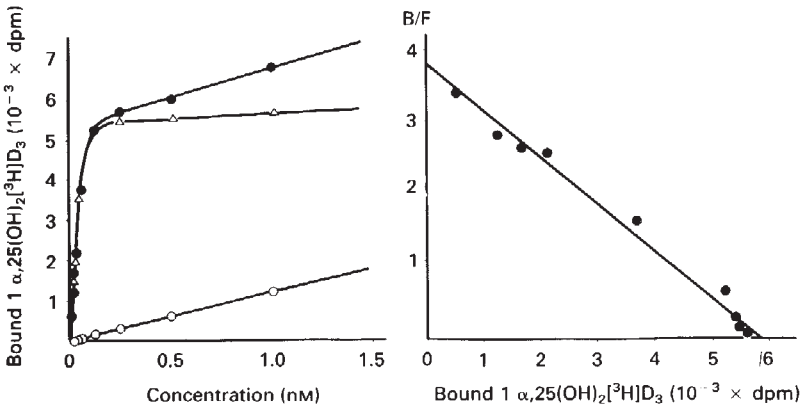

Fig. 5. *Saturation analysis of the receptor in KU-2 cytosol.* Aliquots (500 μliter) of the cytosol (1 mg protein/mliter) were incubated with graded concentrations of $1\alpha,25(OH)_2[^3H]D_3$ in the presence or absence of a 1,000-fold molar excess of radioinert $1\alpha,25(OH)_2D_3$. Symbols are: ●, total binding; ○, non-specific binding; △, specific binding. Data are from the means of triplicate experiments. *Kd* and *Nmax* were derived from three replications of the saturation analysis (mean ± SE). *Kd* = 20.8 ± 1.8 pM. *Nmax* was calculated as approximately 4000 molecules/cell. *Nmax* = 87 ± 24 fmole/mg protein.

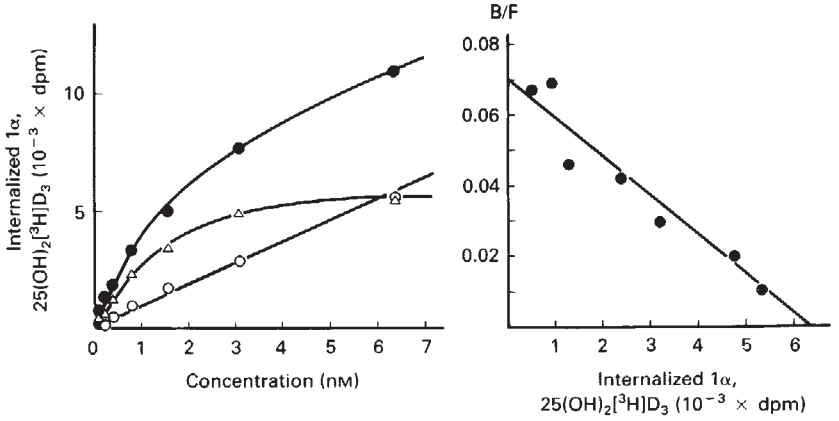

Fig. 6. *Saturation analysis of the internalization of $1\alpha,25(OH)_2[^3H]D_3$ by intact KU-2 cells in the medium supplemented with 10% FBS.* Aliquots of 0.4 mliter of cell suspension (8×10^6 cells, total) were incubated with graded concentrations of $1\alpha,25(OH)_2[^3H]D_3$ in the presence of (○) or absence (●) of a 100-fold molar excess of radioinert $1\alpha,25(OH)_2D_3$ (left). Scatchard plot analysis of the specific internalization (△) showed that the *Kint* was 1.2 nM and the internalized capacity was 33 fmole/8 $\times 10^6$ cells (2500 molecules/cell) (right).

which $1\alpha,25(OH)_2D_3$ internalization (*Kint*) by intact KU-2 cells was 1.2 nM in the same condition as was used in the growth study, which was very close to the half-maximal dose of $1\alpha,25(OH)_2D_3$ at approximately 2 nM, to inhibit growth of KU-2 cells in the monolayer and soft agar cultures. Additionally, the affinity of various vitamin D_3 derivatives in binding to the cytosol receptor of KU-2 cells was closely related to the ability to inhibit proliferation of the cells. Frampton et al [25] reported that a renal cell carcinoma cell line (Colo 293) has an $1\alpha,25(OH)_2D_3$ receptor with a low binding capacity, but the biological functions of the receptor were not described. In other tumor cells, Mangelsdorf et al [5] reported that $1\alpha,25(OH)_2D_3$ inhibited growth of HL-60 cells by a receptor-mediated mechanism. They drew such a conclusion from evidence that the *Kint* of HL-60 cells was virtually identical to the effective dose in inducing 50% differentiation of the cells, and that a relatively receptor-poor blast cell line of HL-60 was insensitive to the induction of differentiation by $1\alpha,25(OH)_2D_3$. Dokoh et al [8] have also demonstrated that $1\alpha,25(OH)_2D_3$ inhibited growth of receptor-containing rat osteogenic sarcoma cells (ROS 17/2.8) more effectively than that of receptor-poor cells (ROS 24/1) in a soft agar culture. This suggested that the growth inhibition by $1\alpha,25(OH)_2D_3$ depends on the number of receptor binding sites in the target cells. These results together with the present study indicate the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ are mediated by the specific $1\alpha,25(OH)_2D_3$ receptor. In contrast, Chandler et al [21] recently reported that $1\alpha,25(OH)_2D_3$ induced the 24-hydroxylase activity of $25(OH)D_3$ in monkey kidney cells (LLC-MK_2) which are deficient in similar $1\alpha,25(OH)_2D_3$ receptors with a high affinity. However, the same research group [26] reported very recently that LLC-MK_2 cells possessed the immunologically same species (64,000 dalton) as the authentic $1\alpha,25(OH)_2D_3$ receptor though its affinity was low, which indicated that the receptor may be likewise involved in inducing 24-hdroxylation in these cells.

The *Kd* value of the cytosol receptor was much smaller than the *Kint* value in KU-2 cells. Similar discrepancies between a cytosol *Kd* and a cell *Kint*, or a concentration necessary for growth suppression, are observed in some other cell lines [4–6, 8]. In case of HL-60 cells, the *Kint* of the cells was reported to be 5.4 nM [5], while the cytosol *Kd* was 23 pM [4]. These differences were explained by the fact that the binding data were obtained in cytosolic extracts in the absence of serum, whereas the effects on cell growth or the *Kint* were examined in studies with serum-supplemented culture medium [8]. The presence of serum lowers the concentration of free-$1\alpha,25(OH)_2D_3$ and interferes with the translocation process of the hormone from the culture medium to the interior of the cells. In contrast, Manolagas et al [23] reported that a *Kd* was not different from a *Kint* in rat osteogenic sarcoma cells, because both data were obtained in the serum-free medium. Also the *Kd* value of the $1\alpha,25(OH)_2D_3$ receptor of human lymphocytes was reported to be almost identical to the half-maximal dose in inhibiting of those proliferation [27, 28]. In the present study on KU-2 cells, the *Kint* was larger than the *Kd*, whereas the internalizing and binding capacity were similar

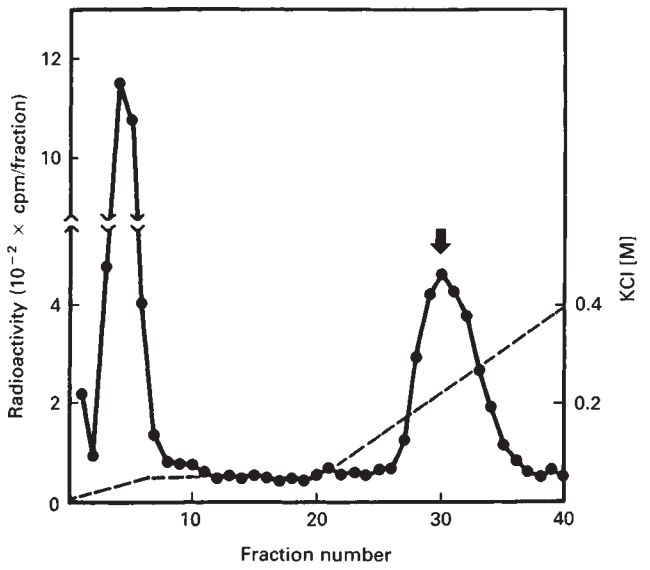

Fig. 7. *DNA-cellulose chromatography of the 1α,25(OH)$_2$[^{3}H]D$_3$ binding in the nuclear extracts of KU-2 cells.* After samples were applied to DNA-cellulose columns, fall-through radioactivity (●–●) was monitored until a consistent baseline was established. Then DNA-binding components were eluted with a linear KCl gradient (---) and the radioactivity was counted. A 1α,25(OH)$_2$[^{3}H]D$_3$-binding component was eluted at a concentration of 0.22 M KCl.

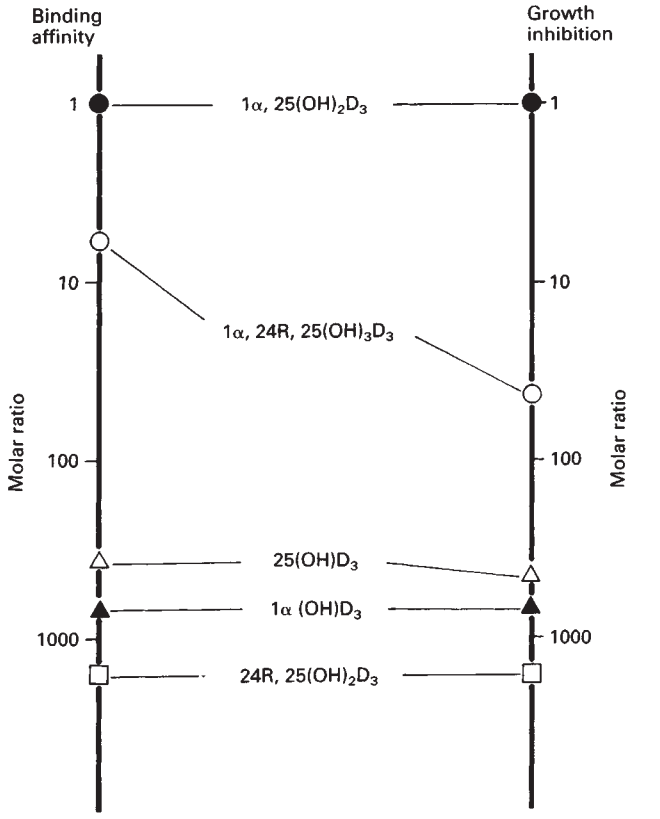

Fig. 8. *A diagram representing the specificity of various derivatives of vitamin D$_3$ in inhibiting KU-2 cell growth and the binding affinity of the derivatives for the specific cytosol receptor of KU-2 cells.* The growth inhibition was expressed as the molar ratio of the vitamin D$_3$ derivatives to 1α,25(OH)$_2$D$_3$ required for suppressing clonogenicity of KU-2 cells by 70% of the control. The binding affinity was expressed as the molar ratio of the vitamin D$_3$ derivatives to 1α,25(OH)$_2$D$_3$ required for 50% displacement of 1α,25(OH)$_2$[^{3}H]D$_3$ from the receptor.

(2500 and 4000 molecules/cell). Additionally, we studied growth suppression by 1α,25(OH)$_2$D$_3$ in the 2% serum medium. The half-maximal inhibition of cell growth in the 2% serum medium was obtained by adding 0.2 to 0.3 nM 1α,25(OH)$_2$D$_3$, which was one-tenth the concentration in the 10% serum medium. Thus it is likely that the discrepancy between the cytosolic *Kd* and the cell *Kint* or the effective dose in inhibiting cell growth is due to the presence of the serum.

It is not known how 1α,25(OH)$_2$D$_3$ affected the growth kinetics of carcinoma cells. Analysis of the cell cycle of KU-2 cells treated with 1α,25(OH)$_2$D$_3$ showed that growth inhibition by the vitamin resulted in a decrease of the cell population in the S phase and in a relative increase of the cell population in the G1 and G2 phases or non-cycling cells. This suggests that growth inhibition by 1α,25(OH)$_2$D$_3$, unlike some anticancer drugs with cytocidal effects, is non-specific to the cell cycle phase, which results in the extension of doubling time or in setting the cells at rest. Furthermore, the clonogenicity of KU-2 cells was not affected by preincubating the cells with 1α,25(OH)$_2$D$_3$. This indicates that the vitamin inhibits proliferation of the cells in a cytostatic manner.

Of the several naturally occurring factors in regulating tumor growth, sex steroids have been extensively studied as inhibitory or stimulative in some particular tumors, such as prostatic carcinoma and mammary carcinoma. These carcinoma cells possess receptors for sex steroids. These steroids have already been utilized as useful and indispensable therapeutic agents. Renal cell carcinoma occurs more frequently in males than in females, with a decrease in the male/female ratio in older patients. It is known that some renal cell carcinomas also have receptors for sex steroids [29, 30]. Chemotherapy using these agents have been established since the middle 1960's as an important therapeutic modality for advanced renal cell carcinoma. The overall response rate to the sex steroid therapy was 6 to 33% (mean, 15%) [31–33]. The result of this study confirms that KU-2 is one of the cell lines in which 1α,25(OH)$_2$D$_3$ exhibits growth inhibition by a receptor-mediated mechanism. Also, the active metabolites of vitamin D suppressed clonogenicity of KU-2 cells, which suggests that the vitamin induces differentiation of the cells or suppresses the tumorigenicity. These consequently indicate that the vitamin might be one of the factors, like sex steroids, in modulating growth and other biological functions of renal cell carcinoma, suggesting further that vitamin D compounds might also be agents for the treatment of renal cell carcinoma.

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